

**UNITED STATES AIR FORCE  
RESEARCH LABORATORY**

**THE ISOLATED PERFUSED RAT LIVER  
AND ITS USE IN THE STUDY OF  
CHEMICAL KINETICS: QUALITY AND  
PERFORMANCE PARAMETERS**

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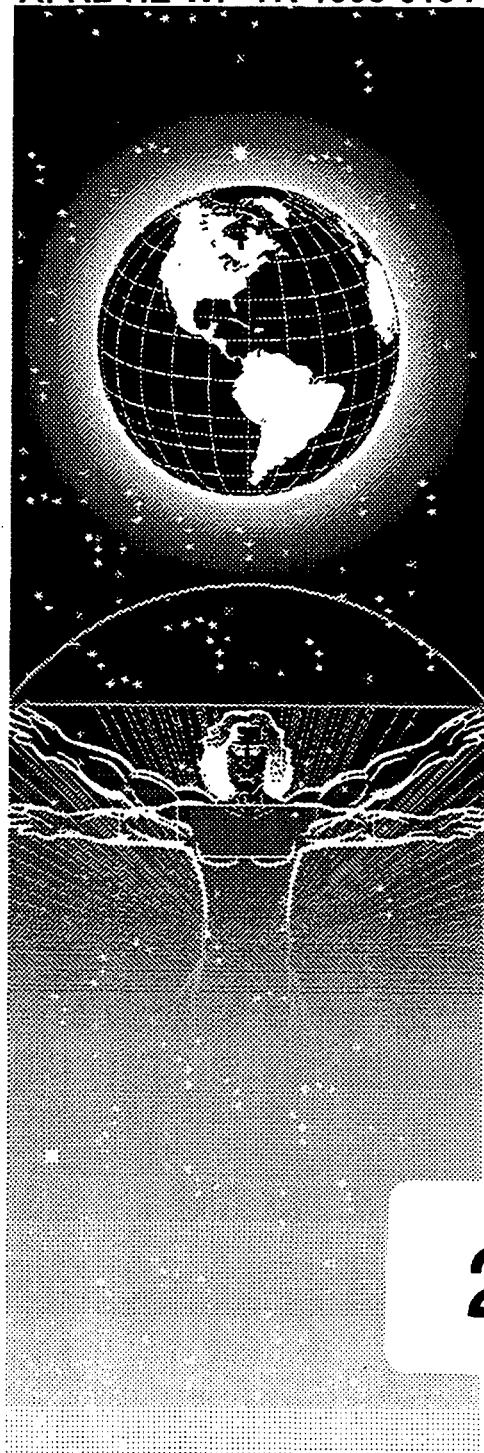
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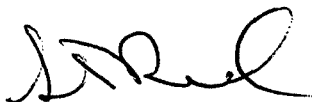
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The animal use described in this study was conducted in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals", National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

**FOR THE DIRECTOR**



**STEPHEN R. CHANNEL**, Maj, USAF, BSC  
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## PREFACE

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## LIST OF ABBREVIATIONS

$A_L$	Activity in liver
$A_{PM}$	Activity in perfusion medium
ATP	Adenosine triphosphate
BBK	Biologically based kinetic
BSA	Bovine serum albumin
BSP	Bromosulfophthalein
$CO_2$	Carbogen dioxide
D	Dry weight
DBSP	Dibromosulfophthalein
Fig	Figure
g	Gram
GSSG	Oxidized glutathione
$H_2O$	Water
IPRL	Isolated perfused rat liver
$K_{EL}$	Elimination rate constant
LDH	Lactate dehydrogenase
$\mu L$	Micro liter ( $10^{-6}$ liter)
mL	Milliliter ( $10^{-3}$ liter)
min	Minute (time)
mM	Millimolar ( $10^{-3}$ molar)
$\mu M$	Micromolar ( $10^{-6}$ molar)
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
nm	Nanometer ( $10^{-9}$ meter)
$O_2$	Oxygen
PE	Polyethylene
$t_{1/2}$	Half-life for elimination
W	Wet weight

# THE ISOLATED PERFUSED RAT LIVER AND ITS USE IN THE STUDY OF CHEMICAL KINETICS: QUALITY AND PERFORMANCE PARAMETERS

## 1.0 INTRODUCTION

### 1.1 The Isolated Perfused Liver

The aim of using the isolated perfused rat liver (IPRL) model in our laboratory is to gain more information on the role of the liver in the *in vivo* kinetics of chemicals. The liver is a major controlling factor in determining systemic kinetics of many chemicals. The IPRL (Bartosek *et al.*, 1972) is a useful *in vitro* system to study the role of the liver in the kinetics and metabolism of chemicals (Ookhtens and Kaplowitz, 1990). The long term goal is to integrate data obtained using the IPRL system into biologically based kinetic (BBK) models to improve the quality of kinetic models and to help reduce the utilization of laboratory animals in the process of risk assessment.

The major advantage of the IPRL system over *in vivo* studies is the elimination of interactions of chemicals with other organs and blood constituents. This provides the ability to control the concentration of chemicals entering the liver. Furthermore, the perfusion medium can easily be manipulated to investigate important mechanistic processes that control *in vivo* kinetics, e.g. to study the role of protein binding in hepatic kinetics. An additional advantage of the IPRL over other *in vitro* systems such as liver homogenates, isolated hepatocytes or liver slices is the possibility of studying the mechanisms of biliary excretion both quantitative and qualitative.

The production of bile is a major physiological/biochemical function of the liver and plays an important role in fat digestion (Reichen and Paumgarten, 1980). In addition, biliary pathways allow for the excretion of endogenous and exogenous chemicals preventing the accumulation of potentially harmful compounds (Klaassen and Watkins, 1984). Both endogenously and exogenously generated compounds and their metabolites are excreted in bile in concentrations that can be up to 1000 times higher than their blood

concentrations. These large concentration gradients support the hypothesis that many biliary transporters are energy (ATP) dependent. Among the compounds excreted in bile are oxidized glutathione (GSSG), glutathione and glucuronide conjugates, bile acids, conjugated bilirubin, and cysteinyl leukotrienes (Coleman, 1987). Due to the important role biliary excretion plays in the elimination of chemicals and their metabolites, the nature of these processes must be considered when predicting the biokinetics of xenobiotics. The IPRL system is a valuable tool in this process.

In addition to the advantage of the ability to study biliary excretion, hepatocytes maintain their normal functional polarity and localisation in the liver lobule (Meijer *et al.*, 1981). Metabolic zonation in the liver has been observed for oxygen consumption (Matsumura *et al.*, 1986), bromosulfophthalein (BSP) metabolism (Chen *et al.*, 1984; Gumucio *et al.*, 1984; Zhao *et al.*, 1993), and benzoic acid metabolism (Chiba *et al.*, 1994). Besides information on the rate of uptake and rate of biliary excretion of a chemical by the liver, potential toxic effects on liver performance can be studied, e.g. the extent of enzyme leakage and alterations in bile flow in the presence of a chemical.

A disadvantage of the IPRL model is that liver viability can only be maintained for 2–4 hours without incurring excessive costs. However, for kinetic studies this time period is often more than sufficient to obtain useful data. A BBK model for the kinetics of water soluble compounds in the IPRL is being developed in this laboratory (Frazier, 1997). This model facilitates the interpretation of kinetic data by integrating the role of membrane transport, protein binding, and metabolism in hepatic kinetics.

## 1.2 The IPRL System at AFRL/HEST

In general, the perfusion cabinet contains a reservoir pump, gas exchange chamber (artificial lung), temperature probe, pH probe, oxygen electrode and pressure transducer. Details on the instrumentation used in the perfusion cabinet are described in addendum 10.1 in Appendix A (SOP NO. PD-97-011). Figure 1 is a diagram of the system that is used in AFRL/HEST as described by Wyman *et al.* (1995). The perfusion medium usually consists of a physiological salt solution (Krebs-Ringer) supplemented with glucose and bovine serum albumin (BSA, referred to as albumin in the text). The Krebs-Ringer medium is CO<sub>2</sub> buffered and pH is maintained by changing the CO<sub>2</sub> : O<sub>2</sub> ratio (default = 5 :

95) in the artificial lung which consists of 25 feet of Silastic tubing in a glass jar continuously gassed with 95% O<sub>2</sub>/ 5% CO<sub>2</sub>. The flow rate of perfusion medium without an oxygen carrier (e.g. without red blood cells) is maintained between 25 – 40 mL/min to ensure adequate oxygenation of the liver. Using higher flow rates results in mechanical damage in the liver sinusoids. The IPRL system that was developed in this laboratory uses perfusion media prepared under sterile conditions, since bacterial endotoxin has been shown to affect responses of the liver to challenges (Wettstein *et al.*, 1995, Sneed *et al.*, 1997). Low-endotoxin albumin is used in the preparation of perfusion medium and its concentration was set at the physiological concentration of albumin in rat blood of 4% (w/v).

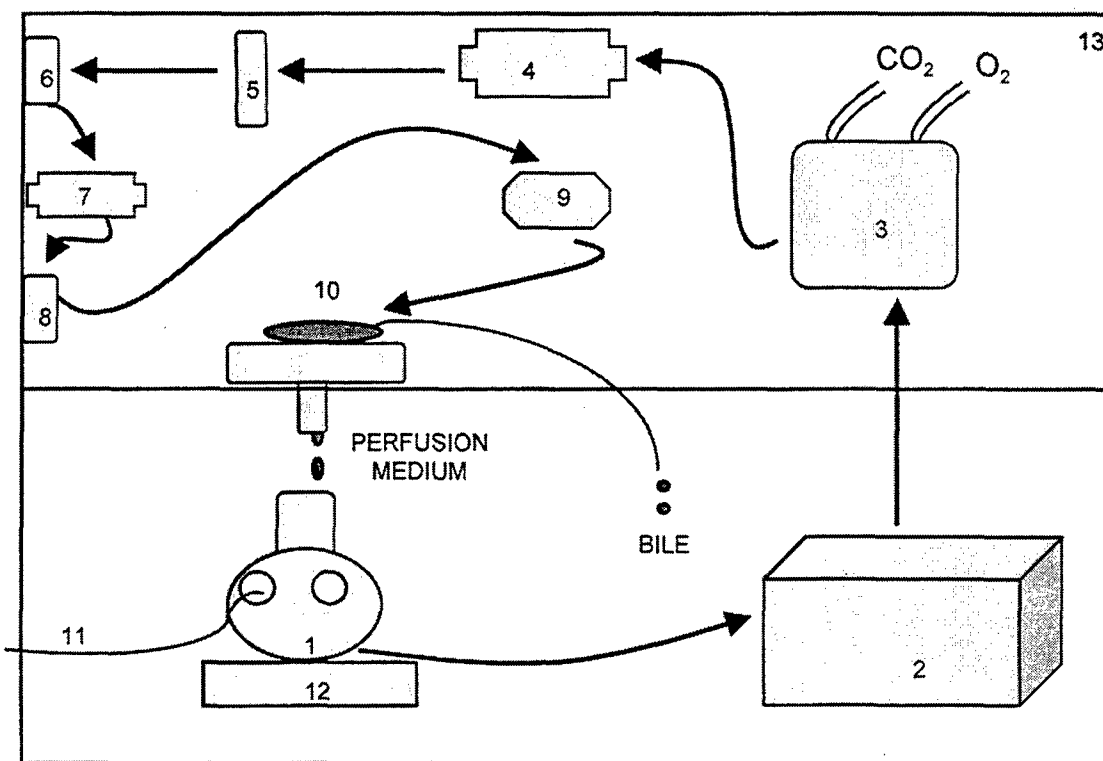


FIGURE 1: SCHEMATIC DIAGRAM OF THE IPRL SYSTEM

Components: 1. Perfusion medium reservoir; 2. Pump; 3. Gas exchange chamber; 4. Temperature probe; 5. Filter; 6. pH probe; 7. Bubble trap; 8. Oxygen electrode; 9. Pressure transducer; 10. Liver; 11. Taurocholate infusion syringe; 12. Magnetic stirrer; 13. Temperature controlled plexiglass box.

In the literature, two different modes of perfusion have been described: the single pass mode versus the recirculating mode. Advantages of using the single pass mode are

that there is a constant concentration of chemical entering the liver and that there is no build-up of (toxic) metabolites in the perfusion medium. Its main disadvantages are the large volumes of perfusion medium needed, which leads to high costs due to the requirement for expensive albumin, and an inability to detect relatively slow kinetic processes. Advantages of using the recirculating mode are its lower cost and the ultimate elimination of chemicals via slow kinetic pathways. The build up of toxic metabolites may not be a problem since experiments are performed over a total time period of only three hours. Actually, the build up of metabolites may be an advantage when the rate of metabolite formation is low. To characterise our system and to have a quality control parameter that can be routinely used, the kinetics of BSP were studied in a recirculating mode. It is important for each IPRL system to establish criteria to assess performance because the use of different flow rates, mode of circulation and buffer constituents makes comparison with data obtained from the literature difficult.

### 1.3 Bromosulphophthalein (BSP)

BSP is routinely used to evaluate liver function, i.e. the rate of BSP disappearance from blood is determined as an indicator of liver performance (Tietz, 1982). Efficient elimination of BSP by the liver requires uptake through the hepatic sinusoidal membrane, glutathione conjugation and active excretion of BSP and its glutathione conjugates in bile through the canalicular membrane. The molecular weight of BSP is 838 Da, well above the threshold weight for biliary excretion (325 Da; Coleman, 1987). The molecular weight of BSP is increased to above 1,000 Da upon formation of mono- and di-glutathione conjugates. BSP belongs to a group of cholephilic organic anions, and therefore can increase bile production at high concentrations.

Cholephilic compounds are excreted in bile in concentrations 10-1000 times greater than their plasma concentration (Coleman, 1987, Klaassen and Watkins, 1984)). Because of this large concentration gradient between plasma and bile, these compounds are thought to be actively excreted by a transporter in the canalicular membrane. Data concerning BSP and protein binding (Weisiger, 1993; Pfaff *et al.*, 1975; Gumucio *et al.*, 1984), plasma membrane transport (Wolkoff, 1993; Tiribelli *et al.*, 1978), liver metabolism and bile excretion (Sathirakul, 1994) are available in the literature. Kinetics of BSP and

BSP analogues have been studied using the IPRL system. These analogues include BSP conjugated with glutathione (cBSP) (Van Dyke *et al.*, 1983) and dibromosulfophthalein (DBSP) (Meijer *et al.*, 1984). Characterising BSP kinetics in our IPRL model will give an indication about the quality and performance of our IPRL preparation.

This report documents bile flow, enzyme leakage and the liver water content of control and BSP-exposed livers. In addition, the kinetics of 20  $\mu$ M BSP in perfusion medium containing 4% albumin in our in-house recirculating IPRL system are presented. The BSP concentration in perfusion medium and bile was determined over a 150 min exposure period.

## 2.0 MATERIALS AND METHODS

### 2.1 Materials

Low endotoxin BSA, taurocholic acid and BSP were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin (1,000 U/mL) was obtained from Solopak Laboratories Inc. (Elk Grove Village, IL). All other chemicals used were of analytical grade.

### 2.2 Animals

Male Fischer 344 rats (230-300 g) were used for liver isolations. Rats had free access to food and water purified by reversed osmosis. Animal use described in this study was conducted in accordance with the principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996, and the Animal Welfare Act of 1966, as amended.

### 2.3 Liver Isolation

Liver surgery was performed as described by Wyman *et al.* (1995). Briefly, rats were anesthetized with diethyl ether to minimize effects on liver function. After cannulating the bile duct with PE-10 tubing, the portal vein was cannulated using an 18-gauge 2-inch catheter. The liver was flushed with 2 mL prewarmed heparin/saline solution (500 U heparin/mL in 0.9% NaCl) and the cannula was secured with a silk ligature. Following severance of the inferior vena cava, the liver was perfused with Krebs-Ringer medium supplemented with 11.5 mM glucose and saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub>, pH 7.40, 37 °C at a flow rate of 25 mL/min. After cannulating the superior vena cava with PE-205 tubing via an incision in the right atrium, the inferior vena cava was closed with a silk ligature and the liver was carefully excised.

## 2.4 Liver Perfusion

The liver perfusion cabinet (Figure 1) was the same as described by Wyman *et al.* (1995). Flow rate of the perfusate was set at 40 mL/min to assure adequate oxygenation of the liver. Krebs-Ringer buffer (200 mL) supplemented with 4% (w/v) low-endotoxin BSA and 11.5 mM glucose was used to perfuse the liver during experiments. To sustain bile production, taurocholate was infused into the perfusion medium at a rate of 33.5 µmoles/h. The medium was oxygenated by passing it through the gas exchange apparatus. Medium pH was maintained between 7.37–7.42 by adjusting the oxygen flow rate, thereby changing the % CO<sub>2</sub> in the gas exchanger. Temperature, pH and the percentage oxygen saturation of the perfusion medium and hydrostatic pressure at the portal vein cannula were constantly monitored. Table 1 summarizes the animal weight of rats used as liver donors, the liver weight, and the water content for the IPRL studies described here. For more details on IPRL procedures see attachment A: SOP NO: PD-97-011 and attachment B: SOP NO: PD-97-009.



## 2.5 Viability Parameters

To evaluate the quality of the livers during perfusion, the following parameters were monitored:

- *Enzyme Leakage*: During kinetic experiments samples of perfusion medium (0.5 mL) were collected every 30 min and the activity of LDH was determined as described by Koreniewski and Callewaert (1983). The percentage LDH-leakage during the experiments was calculated as the percentage of activity in perfusion medium at time  $t$  ( $A_{PM}(t)$ ) relative to the total LDH activity. The total activity is the activity in the perfusion medium at the end of the experiment ( $A_{PM}(120)$ ) plus the activity in liver calculated from the liver homogenate ( $A_L(120)$ ). Percentage leakage =  $100 * A_{PM}(t) / (A_{PM}(120) + A_L(120))$ . For more details see attachment C: SOP NO: PD-97-008 "Enzyme Leakage from the Isolated Perfused Rat Liver".
- *Bile Flow*: Bile was collected in pre-weighed microcentrifuge tubes over intervals of 30 min. Bile flow is expressed as  $\mu\text{L} * \text{min}^{-1} * \text{g liver}^{-1}$ , assuming that the density of bile is 1 g/mL.
- *Liver Water Content*: An aliquot of approximately 1 g minced liver was weighed (wet weight,  $W$ ), dried for 7 days at 120 °C in a vacuum oven and weighed again (dry weight,  $D$ ). The percentage water content was calculated as  $100 * (W-D) / W$ .

## 2.6 Kinetic Studies

After connecting the liver into the perfusion system, the liver was allowed to recover from surgery for 30 min before BSP dosing (designated  $t = 0$ ). To start BSP exposure, an aliquot of 200  $\mu\text{L}$  of a 20 mM BSP stock solution (4  $\mu\text{moles}$  of BSP) in nanopure  $\text{H}_2\text{O}$  was added to 200 mL perfusion medium in the reservoir. This resulted in a medium concentration of 20  $\mu\text{M}$  BSP. To determine the BSP concentration in the perfusion medium ( $C_{\text{PERFUSION MEDIUM}}$ ), samples (0.5 mL) were taken at 2, 5, 10, 20, 30, 60, 90, 120 and 150 min after BSP addition. Bile was collected over intervals of 30 min. All samples were kept on ice until analyzed. At the end of the 150 min exposure, the liver was perfused with 10 mL of chemical free perfusion medium to flush the sinusoids.

After removing the cannula and excess tissue the liver was weighed, cut in pieces and homogenized with 0.9% NaCl (1 g liver : 3 mL saline). To determine the BSP concentration in perfusion medium, 0.5 mL 1 M NaOH was added to 0.5 mL medium. To determine BSP concentration in bile, 10  $\mu$ L bile was added to 1 mL unused perfusion medium and 1 mL 1 M NaOH. BSP standards were prepared in a mixture of 1 mL perfusion medium and 1 mL 1 M NaOH. BSP was determined spectrophotometrically at 580 nm using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Note, this procedure determines the total concentration of BSP and its metabolites. To calculate the concentration of BSP in perfusion medium and bile, a standard curve of BSP absorbance at 580 nm was generated in SIGMA plot (Jandel Scientific). For more details see attachment D: SOP NO: PD-97-010 "Bromosulphophthalein (BSP) Kinetics in the Isolated Perfused Rat Liver".

### 3.0 RESULTS

#### 3.1 Viability Criteria

The activity of LDH in the perfusion medium started to increase slowly 90 min after connecting the liver into the perfusion system. Leakage of LDH in perfusion medium showed a similar pattern for BSP-exposed livers and controls and was approximately 15% at the end of the experiment (Fig 2A). Initial bile flow exceeded 1.0  $\mu$ L\*min<sup>-1</sup>\*g liver<sup>-1</sup> and slowly decreased over time remaining > 0.7  $\mu$ L \* min<sup>-1</sup> \* g liver<sup>-1</sup> at the end of three hours of perfusion (Fig 2B). The liver weight in control groups was slightly less than in BSP groups, but this coincided with slightly lower rat weights (Table 1). The liver weight/body weight ratio was the same for both groups. There was no significant difference in water content between controls and BSP-exposed livers.

TABLE 1: BODY WEIGHT FOR DONOR RATS, LIVER WEIGHT, AND WATER CONTENT FOR THE IPRL STUDIES

Treatment	Rat Weight (g)	Liver Weight (g)	Water Content (%)
Control	241 ± 11	8.6 ± 0.3	79 ± 4
BSP	253 ± 12	9.1 ± 0.3	78 ± 1

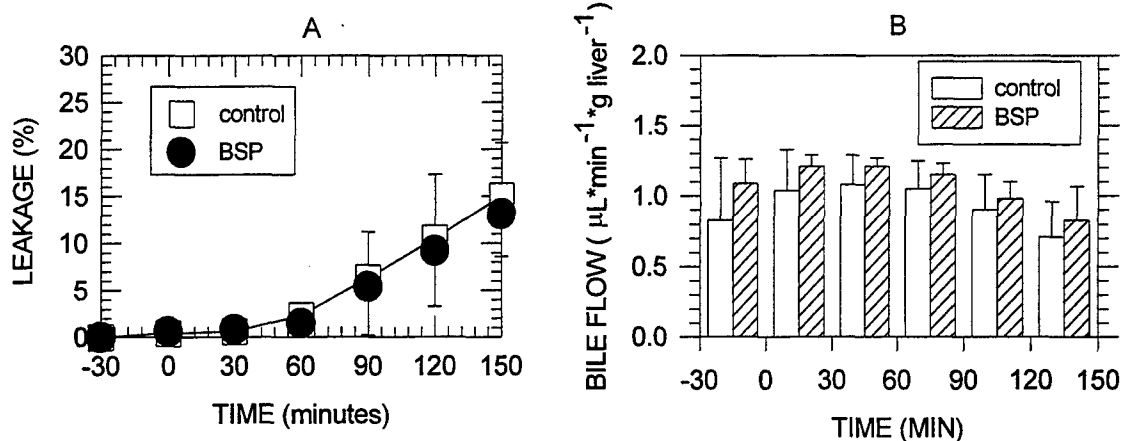


FIGURE 2: VIABILITY OF THE IPRL SYSTEM

The percentage LDH activity in perfusion medium (A) and bile flow in the IPRL ( $\mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ ) (B). Rat livers were perfused for 180 min in a recirculating system. Exposure to 20  $\mu\text{M}$  BSP was started at experimental time  $t=0$ , after a recovery period of 30 min, and the experiment was terminated after 150 min of exposure. Results are expressed as the means  $\pm$  S.D. of 4 separate experiments.

### 3.2 BSP Kinetics

The BSP concentration in the perfusion medium decreased from  $18.2 \pm 1.9 \mu\text{M}$  two min after dosing to  $5.7 \pm 2.7 \mu\text{M}$  after 150 min of exposure (Fig 3A). The total concentration of BSP in bile was markedly elevated relative to the concentration in the perfusion medium and was greater than 100 times that in the perfusion medium at 30 min after BSP exposure started (Fig 3B). This concentration gradient was 200 fold at the end of the experiment. The amount of BSP excreted was calculated by multiplying the volume

of bile produced in a certain time period by the BSP concentration in bile during that period Figure 3C). Of the 4  $\mu$ moles BSP added at the start of the experiment,  $2.3 \pm 0.4$   $\mu$ moles were excreted in bile at the end of the experiment (58% of the initial dose).

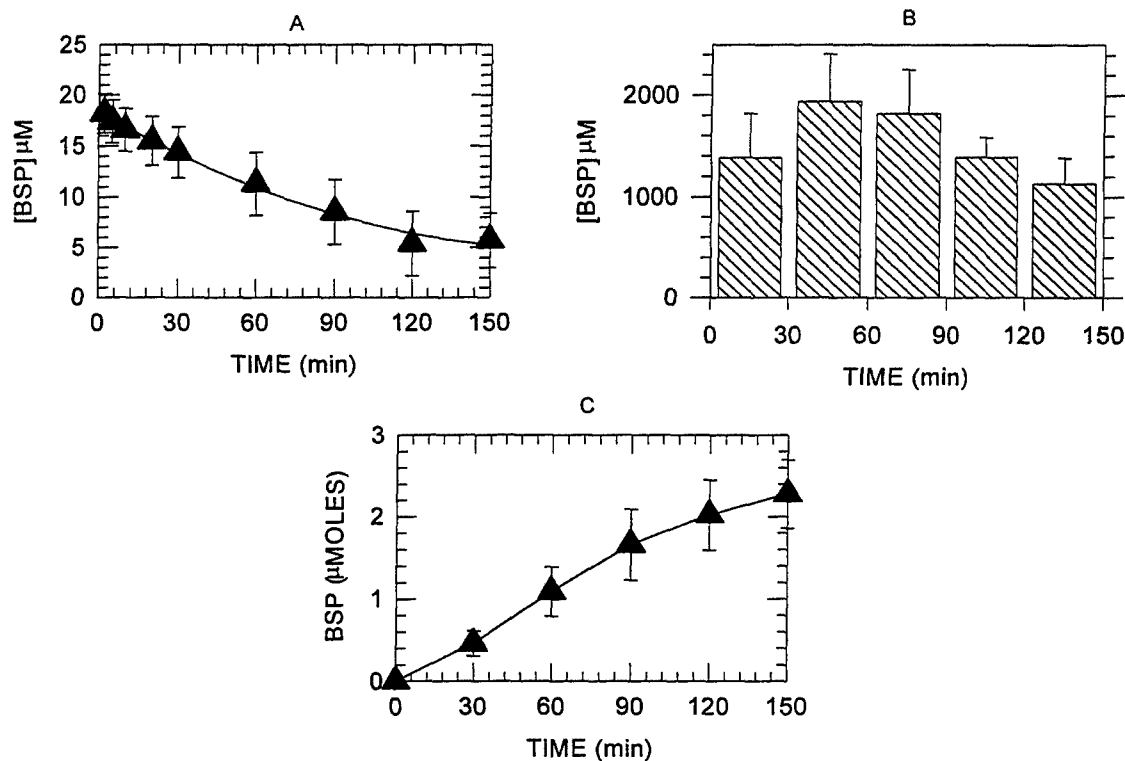


FIGURE 3: KINETICS OF BSP IN THE IPRL SYSTEM

The concentration of BSP in perfusion medium ( $\mu$ M) (A), concentration of BSP in bile ( $\mu$ M) (B) and cumulative excretion of BSP in bile ( $\mu$ moles) (C). Rat livers were treated as in Figure 2.

The apparent first-order elimination rate constant for BSP, calculated from the slope of the curve of  $\log C_{\text{PERFUSION MEDIUM}}$  versus time, was  $K_{\text{EL}} = 8.62 \times 10^{-3} \text{ min}^{-1}$  (Fig 4) corresponding to an half-life for BSP elimination of  $t_{1/2} = 80 \text{ min}$ .

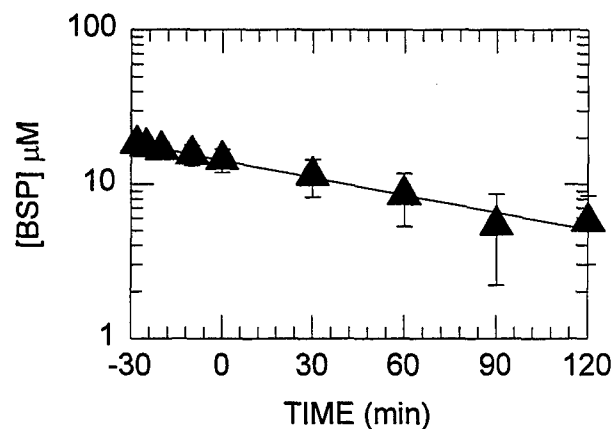


FIGURE 4: LOG  $C_{\text{PERFUSION MEDIUM}}$  VERSUS TIME

Figure 5 illustrates the difference in performance of a poor IPRL preparation (IPRL 67) and a good IPRL preparation (IPRL 133). Poor performance is characterized by an earlier onset in LDH-leakage and, more noticeable, by a lower production of bile in IPRL 67 as compared to IPRL 133 (Fig 5A, B).

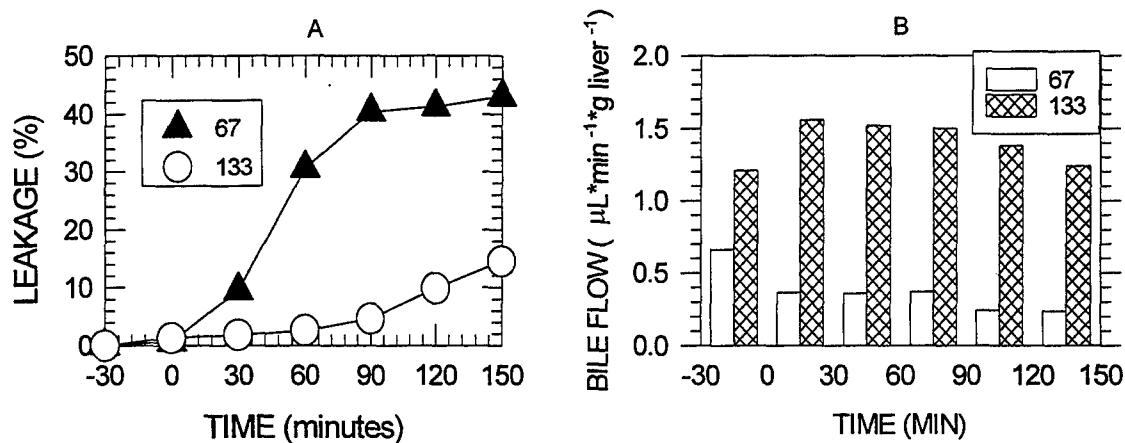


FIGURE 5: VIABILITY OF A POOR IPRL PREPARATION (IPRL 67) AND A GOOD IPRL PREPARATION (IPRL 133)

Percentage LDH activity in perfusion medium (A) and bile flow ( $\mu$ L \* min<sup>-1</sup> \* g liver<sup>-1</sup>) (B). Rat livers were treated as in Figure 2.

Figure 6 shows the difference in BSP kinetics for a poor IPRL preparation and a good one. There is a slight decrease in BSP concentration in perfusion medium in IPRL 67 whereas in IPRL 133 this concentration decreases about 75% (Fig 6A). The biliary concentration of BSP is about three times higher in IPRL 133 as compared to IPRL 67 (Fig 6B). The amount of BSP excreted in bile at the end of the experiment is 2.8  $\mu$ moles for IPRL 133 versus 0.2  $\mu$ moles for IPRL 67 (Fig 6C).

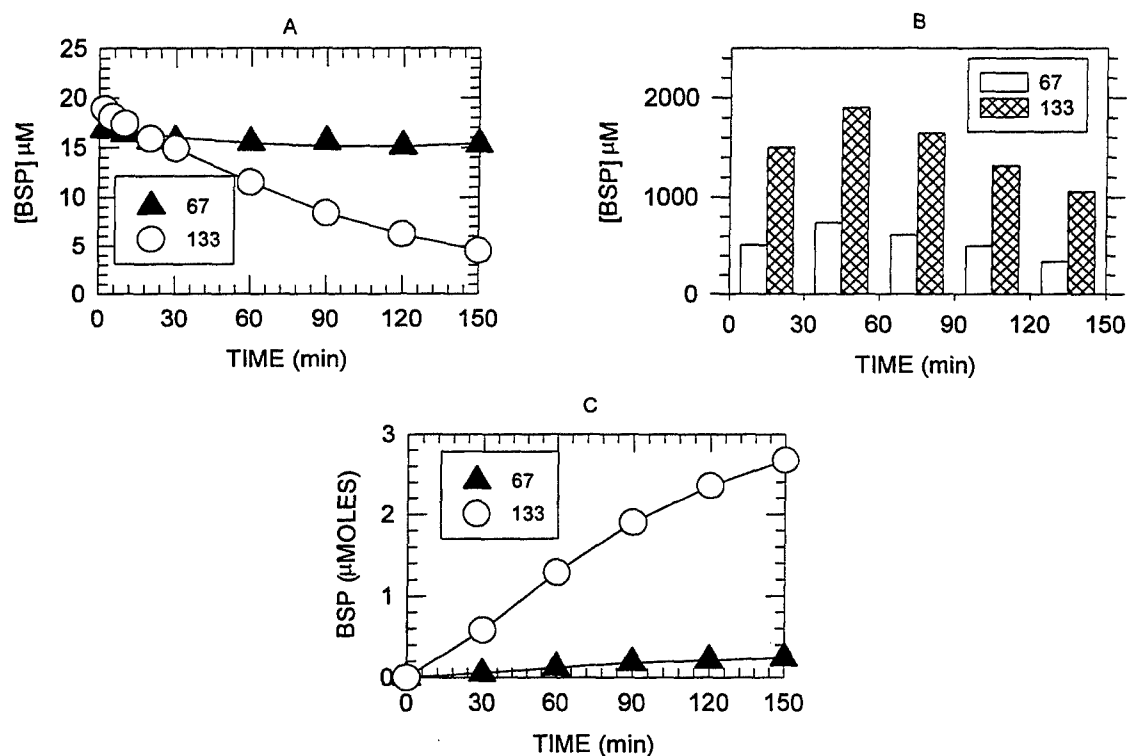


FIGURE 6: KINETICS OF BSP IN A POOR IPRL PREPARATION (IPRL 67) AND A GOOD IPRL PREPARATION (IPRL 133)

The concentration of BSP in perfusion medium ( $\mu$ M) (A), concentration of BSP in bile ( $\mu$ M) (B) and cumulative excretion of BSP in bile ( $\mu$ moles) (C). Rat livers were treated as in Figure 2.

#### 4.0 DISCUSSION

Liver integrity was not severely compromised over the three hour period as indicated by the lack of gross LDH leakage. Moreover, the IPRL performed well throughout the experiments as indicated by the fact that bile flow remained above  $0.7 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$ . There was no effect of BSP treatment on enzyme leakage or bile flow. Good liver performance was also indicated by the maintenance of BSP excretion in bile throughout the experiment. The reason for the lower BSP concentration in bile during the first 30 min of BSP exposure as compared to the second 30 min period is the time delay between dosing at  $t=0$  and the time at which BSP is collected. The time lag, i.e. transit time in bile cannula, is about 6 to 10 min and could be visually observed since the color of control bile is yellow whereas bile containing BSP is purple. At neutral pH BSP is colorless whereas in basic conditions BSP turns purple due to opening of the lactone ring. Therefore, the color change of bile containing BSP shows that bile produced by the IPRL system has a basic pH. This finding agrees with Klaassen and Watkins (1984), who state that bile has a basic pH due to the presence of the bicarbonate anion,  $\text{HCO}_3^-$ .

BSP is a known choleretic compound but did not induce this effect in our studies. The lack of this effect under our conditions is probably due to the low free BSP concentration in perfusion medium. BSP has been shown to have a high affinity for binding to BSA with a  $K_d$  being less than  $0.26 \mu\text{M}$  (Baker and Bradley, 1966; Pfaff *et al.*, 1975; Zhao *et al.*, 1993). Since the BSA concentration in our system is about  $600 \mu\text{M}$ , it is likely that over 99% of the BSP is bound to BSA. An increase in bile production was observed when the IPRL system was exposed to  $200 \mu\text{M}$  of BSP in the absence of albumin (results not shown). A choleretic effect of glutathione conjugated BSP (cBSP) in the IPRL has been reported by van Dyke *et al.* (1983). They used constant infusion rates with concentrations of cBSP ranging from  $30$  to  $314 \mu\text{M}$  cBSP without albumin in perfusion medium. The concentration of BSP in bile and in perfusion medium in our IPRL system is very similar to the cBSP concentrations reported in their study:  $2 \text{ mM}$  versus  $2 \text{ mM}$  in bile and  $20 \mu\text{M}$  versus  $31 \mu\text{M}$  in perfusion medium, respectively. Since van Dyke *et al.* (1983) exposed the IPRL to cBSP, BSP already conjugated with glutathione, this indicates that conjugation is not the rate-limiting step for BSP excretion under our conditions. However, it remains difficult to compare IPRL studies performed in

different laboratories since both the BSP and albumin concentration in perfusion medium significantly affect the ratio between the concentration of BSP in bile relative to its concentration in perfusion medium (Toxopeus *et al.*, 1998). In addition, Toxopeus *et al.* (1998), observed that bile production is lower when the albumin concentration in perfusion medium is decreased.

Currently the IPRL system is used on a routine base in our laboratory and has provided reproducible kinetic data for the kinetics of trichloroacetic acid, dichloroacetic acid, benzoic acid, and BSP, the latter being studied in the presence of various albumin concentrations. The main criterion in our laboratory for liver performance is that bile flow remains above  $1 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  during the first two hours of perfusion and above  $0.7 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  during the last hour. This criterion is only valid when performing experiments with 4% albumin in the perfusion medium. In the presence of lower albumin concentrations bile flow should be between 0.5 and  $0.75 \mu\text{L} \cdot \text{min}^{-1} \cdot \text{g liver}^{-1}$  throughout the experiment. In addition, the onset of enzyme leakage should not occur before 90 min of perfusion. This criterion is independent of albumin concentration. When problems with the IPRL system are suspected, BSP kinetics can be determined to assure that the IPRL system is performing appropriately. BSP kinetics can also be studied in the presence of a test chemical to evaluate the potential toxic effects of that chemical. Adverse effects have been demonstrated in the IPRL using BSP kinetics as a biomarker by peroxisome proliferators (James and Ahokas, 1992 a,b), paracetamol (Khedun *et al.*, 1993), and ischemia-reperfusion injury (Kukan *et al.*, 1995).

## 5.0 CONCLUSIONS

- The present studies indicate that the IPRL system performs well for up to three hours in our laboratory and can be used during this time period to study the kinetics of chemicals.
- The IPRL system can be used to investigate elimination of parent chemical from and appearance of metabolites in the perfusion medium and excretion of parent chemical and metabolites via biliary pathways.



- BSP kinetics can be used as a reliable indicator for liver performance and as a potential indicator of compromised liver performance as a result of chemical exposure.

## 6.0 REFERENCES

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**APPENDIX A: SOP NO. PD-97-011 "Isolated Perfused Rat Liver (IPRL)"**

**AUTHOR:** \_\_\_\_\_

**GA APPROVAL:** \_\_\_\_\_

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**ISOLATED PERFUSED RAT LIVER (IPRL)**

**PURPOSE/PRINCIPLE:**

Purpose: The isolated perfused rat liver is a useful system to study the role of the liver in kinetics and/or metabolism of chemicals. By using an isolated liver there will be no interference of blood constituents and other organs with the compounds studied. The advantage over using isolated hepatocytes is that the liver has an intact architecture and the different cell types making up the liver can still interact with each other.

Principle: The liver is isolated from the rat and maintained in a cabinet for about 3 hours. Bile produced over that period is collected and perfusion medium can be sampled

KEY WORDS: liver, rat, kinetics, metabolism, bile, PD

**1. SAFETY AND OPERATING PRECAUTIONS:**

- 1.1 Wear protective gloves and lab coat during procedures.
- 1.2 Take care when anesthetizing rats with ether. Work in the hood and limit exposure to ether as much as possible.

**2. EQUIPMENT/MATERIALS:**

- 2.1 For equipment used in the perfusion system see attachment 10.1.
- 2.2 Surgery:
  - 2.2.1 Polyethylene tubing PE-10 (Clay Adams, no.7401)
  - 2.2.2 Polyethylene tubing PE-205 (Clay Adams, no. 7446)
  - 2.2.3 Needle, 23-gauge 3/4-inch (Beckton Dickinson)
  - 2.2.4 Catheter, 18-gauge 2-inch (Angiocath)
  - 2.2.5 Syringe, 3 CC

**AUTHOR:** \_\_\_\_\_

**GA APPROVAL:** \_\_\_\_\_

- 2.2.6 Silk ligatures, 5
- 2.2.7 Cotton swabs
- 2.2.8 Gauze (4 x 4)
- 2.2.9 Two pairs of scissors, three pairs of hemostats, one spatula, two pairs of tweezers.

**2.3 Computer:**

- 2.3.1 Neat-J, 3-86, 200 MB hard drive

**2.4 Cabinet:**

- 2.4.1 Oxygen electrode membrane: Yellow Springs Instruments, no. 5775
- 2.4.2 Syringe 10 mL

**3. SPECIMEN:**

- 3.1 Rat: Fisher 344, male, 200-300 g

**4. REAGENTS:**

**4.1 General:**

- 4.1.1 Alcohol  
100% ethanol: 70 mL  
QS to 100 mL with RO water
- 4.1.2 RO water
- 4.1.3 Nanopure water, sterilized over an 0.22  $\mu$  filter
- 4.1.4 Carbogen: 95% oxygen (O<sub>2</sub>) / 5% carbon dioxide (CO<sub>2</sub>)

**4.2 Surgery:**

**AUTHOR:** \_\_\_\_\_

**GA APPROVAL:** \_\_\_\_\_

- 
- 4.2.1 Krebs Ringer Bicarbonate (KRB) medium (see SOP PD-97-009).
  - 4.2.2 Saline - 0.9%  
Sodium Chloride (NaCl) 9 g  
Dissolve in 1 L nanopure water/sterilize over 0.22  $\mu$  filter.
  - 4.2.3 Heparin-1000 units/mL: Solopak Laboratories Inc., no. N 39769-019-10.
  - 4.3 Cabinet:
    - 4.3.1 KRB medium + 4% Bovine Serum Albumin (BSA)
    - 4.3.2 Oxygen probe solution: Yellow Springs Instruments, no. 5775
    - 4.3.3 Buffer solution pH 7.00
    - 4.3.4 Buffer solution pH 10.00
    - 4.3.5 Oxygen
    - 4.3.6 Carbogen (95% oxygen [O<sub>2</sub>] 5% carbon dioxide [CO<sub>2</sub>])
    - 4.3.7 Taurocholate (33.5 mM): Sigma, no. T4009  
Taurocholate 180 mg  
Dissolve in 10 mL nanopure water  
Fill 5 mL syringe with taurocholate and store in freezer. Store rest of taurocholate in fridge at 4 °C.
    - 4.3.8 BSA, low endotoxin: Sigma, no. A 2934
    - 4.3.9 Phosphate Buffered Saline (PBS), 10 mL ice-cold.

5. **PROCEDURE:** (Note: see Addendum 10.2 for checklist)

- 5.1 Setup:
  - 5.1.1 Turn on the 3 power switches to allow warming up of cabinet and water bath.

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GA APPROVAL: \_\_\_\_\_

- 
- 5.1.2 Put KRB media and buffer solutions pH 7 and 10 in 37 °C water bath to warm up.
  - 5.1.3 Thaw syringe filled with taurocholate at room temperature.
  - 5.2 Computer:
    - 5.2.1 The data collection program is initialized using the following sequence of steps:
      - 5.2.1.1 At the C:\ prompt, type g-  
5.2.1.2 Enter experiment no.  
5.2.1.3 Enter flow rate value (40 mL/min)
  - 5.3 Cabinet:
    - 5.3.1 Connect tubing (see Addendum 10.5) and rinse perfusion circuit with 70% ethanol.
    - 5.3.2 Connect medium reservoir and rinse system twice with 150 mL sterile nanopure water.
    - 5.3.3 While rinsing with sterile nanopure water, check flow rate (40 mL/min).
    - 5.3.4 Replace oxygen electrode membrane as described in accompanying manual (Y.S.I.).
    - 5.3.5 Calibrate pH electrode at pH 7.00 (knob: calibration) and 10.00 (knob: slope) with buffer at 37 °C (buffer warmed up in water bath used for surgery).
    - 5.3.6 Put oxygen electrode and pH electrode in position in the flow circuit.
    - 5.3.7 Rinse system twice with 150 mL sterile nanopure water.
    - 5.3.8 Add desired amount of perfusion medium (200 mL) in the reservoir, open the carbogen flow (flow =1.5 L/min) and recirculate medium at least 30 min to allow equilibration of pH and temperature.



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- 5.3.9 Pull pH electrode out and put back in perfusion circuit, debubble tubing. Make a 200 mL mark on the medium reservoir (to compensate for spilled medium later on).
  - 5.3.10 Fill 2 humidity dishes with water and place one dish in the lower and one in the upper part of the cabinet.
  - 5.3.11 Install bile salt infusion system (taurocholate syringe), make sure there is no air in the syringe, check settings (1 mL/h).
  - 5.3.12 Beakers (2) filled with sterile saline (ca. 25 mL) placed in lower cabinet for warming.
  - 5.3.13 Place 3 mL syringe filled with 50/50 heparin/saline with 2-inch 18 gauge angiocath in lower cabinet for warming.
  - 5.3.14 Prepare humidity chamber.
  - 5.3.15 Debubble tubing, add magnetic stirrer in medium reservoir, tilt pressure valve to trap air.
  - 5.4 Surgery:
    - 5.4.1 Sterilize tubing by recirculating 70% ethanol during 2 min.
    - 5.4.2 Flush tubing twice with 150 mL sterile nanopure water.
    - 5.4.3 Fill medium reservoir of surgery system with 450 mL KRB buffer.
    - 5.4.4 Open carbogen flow (connect carbogen tubing; flow rate ca. 0.5 L/min) and recirculate medium at a flow rate of 25 mL/min to allow equilibration of pH and temperature.
    - 5.4.5 Bile tube PE-10 set out (18 inch with beveled end marked with ink).
    - 5.4.5 Vena cava tube with blacked tip set out.
    - 5.4.6 Within reach: needle 23-gauge, cotton swabs, 4 x 4 gauze, silk ligatures, emergency ventilation.

- 
- 5.4.7 Fill ice buckets and put sampling tubes on ice.
  - 5.4.8 Record baseline values for oxygen, temperature, pH and pressure.
  - 5.4.9 Take desired time -60 samples.
  - 5.5 Surgical procedure:
    - 5.5.1 Ether anesthesia
    - 5.5.2 Weigh rat, record weight on IPRL form, and shave rat.
    - 5.5.3 Maintain ether anesthesia of rat by leaving its nose in a cone filled with gauze and ether (be aware of the possibility of ether overdosing).
    - 5.5.4 Sterilize surgical field with 70% alcohol.
    - 5.5.5 Xiphoid-pubis midline incision along the *linea alba* with lateral flaps (clip with hemostats).
    - 5.5.6 Intestines displaced gently to the left in moist gauze (use pre-warmed saline).
    - 5.5.7 Cannulate bile duct by closing the low part of the duct with a ligature, piercing a hole with a 23-gauge needle, and inserting PE-10 tubing. Secure tubing with a silk ligature (make sure bile is still flowing).
    - 5.5.8 Place 1 loose ligature around portal vein and 1 around inferior vena cava above the right renal vein.
    - 5.5.9 Cannulate portal vein using 18-gauge angiocath, extend catheter (be careful not to make a hole with the needle) and slowly inject 3 mL prewarmed heparin/saline mix and withdraw needle.
    - 5.5.10 Secure tip of cannula with ligature and connect medium at lowest flow rate (5 mL/min).
    - 5.5.11 Cut inferior vena cava below the ligature and increase flow rate to 25 mL/min.

- 
- 5.5.12 Free retrogastric lobe (small lobe under stomach) carefully.
  - 5.5.13 Bilateral wide chest incisions and display heart.
  - 5.5.14 Cannulate the superior vena cava via an incision in the right atrium, secure with ligature.
  - 5.5.15 Close ligature around inferior vena cava.
  - 5.5.16 Excise liver carefully and wash surface with warmed saline.
  - 5.5.17 Position liver correctly on hand: look for twisted lobes, check medium outflow.
  - 5.5.18 Transfer liver to cabinet and connect into circuit.
  - 5.5.19 Optimize position of liver to ensure lowest possible pressure (40-60) (this is critical!).
  - 5.5.20 Install humidity chamber over liver.
  - 5.5.21 Turn bile salts pump on (1 mL/h).
  - 5.6 Experiment:
    - 5.6.1 Start timer and observe temperature and pH carefully.
    - 5.6.2 Increase oxygen flow if pH decreases (pH:  $7.40 \pm 0.04$ ).
    - 5.6.3 Clean up surgery table.
    - 5.6.4 Add KRB medium + BSA to restore 200 mL volume 15 min after connecting liver in cabinet.
    - 5.6.5 Let liver equilibrate at least 30 min before starting experiments.
    - 5.6.6 Make a note of pressure, pH, temperature, and % oxygen every 30 min.

- 5.6.7 At end of experiment, flush liver through cannula using a syringe filled with 10 mL ice cold PBS, remove liver from cabinet and cut cannula, diaphragm and fascia from liver.
- 5.6.8 Blot dry and weigh (record weight on IPRL form).
- 5.6.9 Collect perfusion medium in a 250 mL volumetric cylinder and record volume on IPRL form (subtract 10 mL to compensate ice cold PBS).
- 5.7 Clean up:
  - 5.7.1 Surgery:
    - 5.7.1.1 Close carbogen and oxygen flow and switch of 2 right power switches.
    - 5.7.1.2 Flush tubing with 0.5 L water.
    - 5.7.1.3 Wash surgical instruments with soap and rinse with RO water.
    - 5.7.1.4 Neutralize biological fluids with 10% bleach and dispose down drain.
    - 5.7.1.5 Dispose remains of rat in biological waste container.
  - 5.7.2 Cabinet:
    - 5.7.2.1 Flush perfusion system with 400 mL RO water.
    - 5.7.2.2 Rinse oxygen electrode and store in a beaker filled with RO water.
    - 5.7.2.3 Rinse pH electrode with RO water and store in pH 4.00 buffer solution.
    - 5.7.2.4 Close the oxygen and pH electrode gaps in the system and flush system with 200 mL soap solution.
    - 5.7.2.5 Flush system with 2 L RO water, disassemble tubing.
    - 5.7.2.6 Soak filter, pressure transducer, medium reservoir, temperature probe holder, and plateau in soap and rinse with water, dry.

## 6. DATA ANALYSIS/RECORDS:

- 6.1 Calculations:

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- 6.1.1 Viability criteria: results for the parameters of liver viability are shown in the attachment. These parameters are: enzyme leakage (SOP No. PD-97-008), bile flow and % water in liver. Results shown are from control (= non-exposed) livers (addendum 11.6).
- 6.1.2 Kinetic data: dependent on experiment (for examples see SOPs on BSP (SOP No. PD-97-010) and TCA (no. PD-97-012) kinetics in the IPRL)
- 6.2 Results :
  - 6.2.1 See SOPs referred to in previous paragraph (6.1.1)

## 7. QUALITY CONTROL:

- 7.1 There are several ways to check for the quality of the liver:
  - 7.1.1 Liver functioning: bile flow  $> 1 \mu\text{L min}^{-1} \text{g}^{-1}$  during first 2 hours.
  - 7.1.2 Liver integrity: enzyme leakage (lactate dehydrogenase/aspartate transferase/ALT) /  $\text{K}^+$  leakage (low enzyme leakage during first 1.5 hours, increasing to maximal 20-25% at end experiment).
  - 7.1.3 Histopathology:
  - 7.1.4 %  $\text{H}_2\text{O}$

## 8. LIMITATIONS OF PROCEDURE: N/A

## 9. PROCEDURE NOTES:

- 9.1 Will indicate circumstances that would vary from outlined procedure.

**10. REFERENCES:**

- 10.1 Wyman J, Stokes JS, Goehring M, Buring M and Moore T. Data collection interface for isolated perfused rat liver: recording oxygen consumption, perfusion pressure, and pH. *Toxicology Methods* 5: 1-14, 1995.

**11. ADDENDA/ATTACHMENTS:**

- 11.1 System
- 11.2 Check list
- 11.3 Experiment log
- 11.4 Experiment page
- 11.5 Cabinet setup
- 11.6 Enzyme leakage (A, B) and bile flow (C) in control isolated perfused rat liver preparations (N=4)

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## **ADDENDUM 10.1**

### **SYSTEM**

#### **SURGERY:**

1. Pump: Cole Parmer, WZIR057
2. Pump head: Cole Parmer, 7014-10
3. Gas flow regulator: Dwyer, S627
4. Water Bath: GCA/Precision Scientific, model 181

#### **COMPUTER**

1. Neat-J, 3-86, 200 MB hard drive

#### **CABINET**

1. Pump: Harvard, model 55 77-66 (2)\*
2. Gas exchange tubing (25 feet): Dow Corning Silastic tubing 0.147-cm i.d., 0.196-cm o.d. (3)
3. Temperature-sensitive electrode: Yellow Springs Instruments, no. 403 (4)
4. Temperature controllers: Yellow Springs Instruments, model 63 RC
5. Filter: Millipore, stainless-steel filter screen (5)
6. pH electrode: Orion, model 910600 (6)
7. pH electrode monitor: Orion, model 64
8. Bubble trap (inverted "Y") (7)
9. Oxygen electrode: Yellow Springs, model 5775 (8)
10. Oxygen electrode monitor: Yellow Springs, model 5300
11. Pressure transducer: Hewlett Packard, model 1290 C (9)
12. Syringe pump: Orion, model 341B (11)
13. Magnetic stirring plate: Variomag Electronic rührer compact HP1 (12)
14. Magnetic stirring bar
15. Temperature probes
16. Heating elements
17. Fans: Paomotor Miniature Fan, model 3000
18. Gas flow regulator (oxygen): Dwyer, model S629
19. Gas flow regulator (carbogen): Dwyer, model S627

\* numbers in parenthesis refer to numbers in Addendum 10.5.

## **ADDENDUM 10.2 - CHECK LIST**

### **ISOLATED PERFUSED RAT LIVER EXPERIMENTAL CHECKLIST**

#### **1.0 PRELIMINARY SETUP (DAY BEFORE PERFUSION)**

- 1.1 Krebs Ringer Bicarbonate medium (see SOP #4300-4056) mixed and filtered (0.22 m, sterile).
- 1.2 Sample tubes prepared as necessary.
- 1.3 Prepare forms for pathology, enzymes and surgery.
- 1.4 Check gas tanks.

#### **2.0 COMPUTER PROGRAM OPERATION**

- 2.1 Initiate data logging program.

#### **3.0 CABINET SETUP**

- 3.1 Calibrate pressure transducers.
- 3.2 Recirculate system with 70% ethanol for 5 min.
- 3.3 Wash system 2 times with 200 mL distilled, sterile water and check perfusion flow rate (40 mL/min).
- 3.4 Calibrate pH electrode at 37 °C, rinsed and installed.
- 3.5 Install fresh O<sub>2</sub> probe membranes.
- 3.6 Wash system 2 times with 200 mL distilled, sterile water.
- 3.7 Check temperature controller setting (37 °C).
- 3.8 Add 200 mL filtered perfusion medium to reservoir.
- 3.9 Carbogen gas flow = 1.5 LPM.
- 3.10 Calibrate O<sub>2</sub> electrodes at operational flow rate and temperature (95% O<sub>2</sub>).
- 3.11 Humidity dishes filled and placed in each chamber.
- 3.12 Bile salts infusion system setup (1 mL/h).



#### **4.0 SURGICAL SETUP**

- 4.1 Surgical instruments clean and set out: 3 hemostats, 2 pairs of scissors, tweezers, spatula.
- 4.2 Sterile saline (0.90% w/v) warming in the lower cabinet.
- 4.3 Bile tube PE-10 set out (18 inch with beveled end marked with ink).
- 4.4 Bile collection tubes weighed and installed.
- 4.5 Vena cava catheter with blacked tip set out.
- 4.6 3-mL syringe with 2-inch 18-gauge angiocath filled with 50/50 heparin/saline placed in lower chamber for warming.
- 4.7 IV set installed and washed with 70% ethanol and 300 mL sterile water.
- 4.8 Flush IV set with Krebs medium.
- 4.9 Check perfusate pH.
- 4.10 Debubble tubing.
- 4.11 Ensure adequate cotton tipped swabs, ligatures, and 4 X 4s are available.
- 4.12 Patient emergency ventilation assistance apparatus readily accessible.
- 4.13 Carbogen gas flow = 0.5 LPM.

#### **5.0 SURGERY/EXPERIMENT**

- 5.1 Rat weight: \_\_\_\_\_.
- 5.2 Prepare rat for surgery. **Time:** \_\_\_\_\_.
- 5.3 Transfer liver to perfusion cabinet. **Time:** \_\_\_\_\_.
- 5.4 Turn bile salts pump on.
- 5.5 Start timer.
- 5.6 Start data logging (F5).
- 5.7 Check bile collection system and check for flow.

5.9 Observe pressure, pH, and O<sub>2</sub> readings and note these values every 30 min. Make necessary adjustments.

5.10 Switch off gas supply for IV set.

5.11 Collect samples of PM at scheduled sampling times.

## **6. POST-EXPERIMENT PROCEDURES**

6.1 Hit F10.

6.2 Stop pump.

6.3 Flush liver with 10 mL ice cold PBS.

6.4 Record liver weight \_\_\_\_\_.

6.5 Take slices for light microscopic pathology evaluation.

6.6 Take slices for electron microscopic pathology evaluation.

6.7 Take sample ( $\pm 1$  gram) for wet/dry weight measurements.

6.8 Prepare tissues for biochemical analyses.

## **7. CLEANUP**

7.1 Rinse system with 2-3 L distilled water.

7.2 Check if gas tanks are closed.

7.3 Leave oxygen electrodes in a beaker with distilled water and pH electrode rinsed and in cap.

\* If possible, soak glassware overnight in soap and rinse well with tap water followed by distilled water.

\*\* Oxygen electrodes must be stored dry and covered by membrane (no oxygen probe solution) when not used for longer periods.

### ADDENDUM 10.3 - EXPERIMENT LOG

## EXPERIMENT LOG

EXPERIMENT NO \_\_\_\_\_

DATE \_\_\_\_\_

time min	Press <sub>in</sub> mm Hg	Press <sub>out</sub> mm Hg	OX <sub>in</sub> %	OX <sub>out</sub> %	OX <sub>consumed</sub> μL/min	pH	temp ° C
baseline							
-60							
-30							
0							
30							
60							
90							
120							

[illegible]

# **ADDENDUM 10.4 - EXPERIMENT PAGE**

EXPERIMENT NO: \_\_\_\_\_

PATHOLOGY #: \_\_\_\_\_

DATE: \_\_\_\_\_

RAT NUMBER: \_\_\_\_\_

RAT WEIGHT: \_\_\_\_\_

LIVER WEIGHT: \_\_\_\_\_

SURGERY TIME: \_\_\_\_\_

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OBJECTIVE:

COMMENTS:

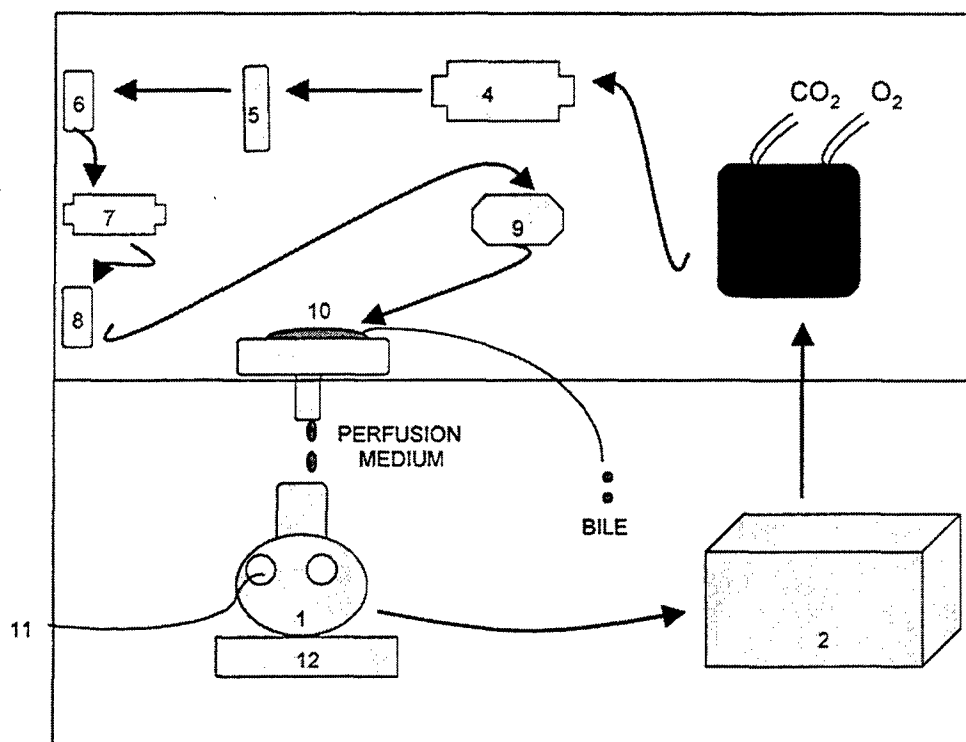
MEDIUM VOLUME: \_\_\_\_\_

WET/DRY WEIGHT: EMPTY VIAL: \_\_\_\_\_

VIAL + LIVER: \_\_\_\_\_

## ADDENDUM 10.5 - CABINET SETUP

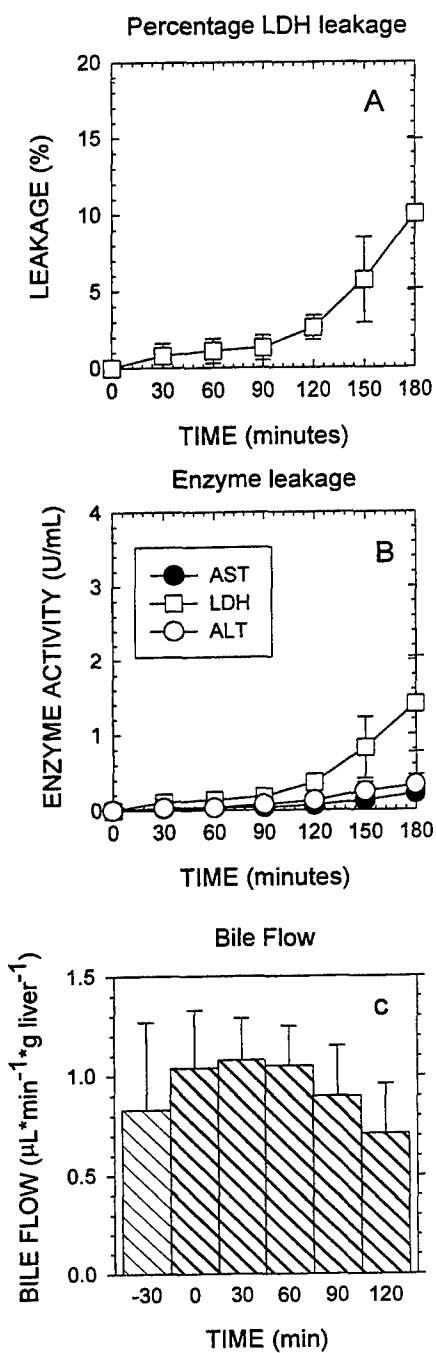
### PERFUSION CABINET



1. Perfusion medium reservoir
2. Pump
3. Gas exchange chamber
4. Temperature probe
5. Filter
6. pH probe
7. Bubble trap
8. Oxygen electrode
9. Pressure transducer
10. Liver
11. Taurocholate infusion syringe

### ADDENDUM 10.6

#### Enzyme leakage and bile flow in control isolated perfused rat liver preparations (N=4)



APPENDIX B: SOP NO. PD-97-009 "Preparation of Krebs Ringer Bicarbonate Buffer for the  
Isolated Perfused Rat Liver"

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**PREPARATION OF KREBS RINGERS BICARBONATE BUFFER FOR THE  
ISOLATED PERFUSED RAT LIVER**

**PURPOSE/PRINCIPLE:**

Purpose: Krebs Ringer buffer is used in experiments with the isolated perfused rat liver (IPRL). The buffer is used during isolation of the liver and buffer containing 4 g Bovine Serum Albumin (BSA)/100 mL is used as perfusion medium during experiments performed with the IPRL.

Principle: This buffer resembles physiological salt concentrations and will sustain liver functioning during experiments with isolated rat liver.

**KEY WORDS:** IPRL, PD, Ringer, buffer

**1. SAFETY AND OPERATING PRECAUTIONS:**

- 1.1 Solutions prepared do not contain hazardous chemicals.
- 1.2 It is recommended to wear a labcoat and gloves.
- 1.3 Work in a biohazard hood to keep stock solutions sterile and prevent contamination of the Krebs Ringer buffer as much as possible.

**2. EQUIPMENT/MATERIALS:**

- 2.1 Stock solutions are prepared in nanopure water as described in SOP NO. 4300-4056 (ManTech). All solutions are sterilized after preparation using 0.22  $\mu$ M filters.
- 2.2 The preparation of the following stock solutions is described in Section 4 of this SOP:



## Standard Operating Procedure

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Date: 27 February 1997

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2.2.1 0.770 M Sodium Chloride (NaCl)

2.2.2 0.770 M Potassium Chloride (KCl)

2.2.3 0.545 M Calcium Chloride (CaCl<sub>2</sub>)2.2.4 0.770 M Potassium Dihydrogen Phosphate (KH<sub>2</sub>PO<sub>4</sub>)2.2.5 0.770 M Magnesium Sulfate (MgSO<sub>4</sub>)2.2.6 0.154 M Sodium Carbonate (NaHCO<sub>3</sub>)

2.2.7 0.3 M Glucose

2.3 Filter sterile using 500 mL and 1 L units (Nalgene).

## 3. SPECIMEN: N/A

## 4. REAGENTS:

4.1 Sample preparation (SOP. NO. 4300-4056 [ManTech]):

4.1.1	NaCl	58.44 g/mol	45 g/L	→ 0.770 M
4.1.2	KCl	74.55 g/mol	28.8 g/500 mL	→ 0.770 M
4.1.3	CaCl <sub>2</sub> * 2H <sub>2</sub> O	147 g/mol	20.2 g/250 mL	→ 0.545 M
4.1.4	KH <sub>2</sub> PO <sub>4</sub> (an)	136.1 g/mol	26.2 g/250 mL	→ 0.770 M
4.1.5	MgSO <sub>4</sub> *7H <sub>2</sub> O	246.3 g/mol	47.4 g/250 mL	→ 0.770 M
4.1.6	NaHCO <sub>3</sub>	84 g/mol	12.9 g/L	→ 0.154 M
4.1.7	Glucose	180 g/mol	10.8 g/200 mL	→ 0.3 M

4.2 Store stock solution in the refrigerator at 4 °C.

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**5. PROCEDURE:**

5.1 Mix solutions in numerical order to avoid precipitation of  $\text{CaCO}_3$ .

# added	stock solution	volume (mL)	end concentration
1	0.770 M NaCl	99.6	118 mM NaCl
2	0.770 M KCl	4.2	5 mM KCl
3	0.545 M $\text{CaCl}_2$	3.0	2.5 mM $\text{CaCl}_2$
4	0.770 M $\text{KH}_2\text{PO}_4$	1.0	1 mM $\text{KH}_2\text{PO}_4$
5	0.770 M $\text{MgSO}_4$	1.0	1 mM $\text{MgSO}_4$

Add 400 mL nanopure  $\text{H}_2\text{O}$ .

# added	solution	volume (mL)	end concentration
6	0.154 M $\text{NaHCO}_3$	105	25 mM $\text{NaHCO}_3$
7	0.3 M glucose	25	11.5 mM glucose

5.2 QS to 650 mL with nanopure  $\text{H}_2\text{O}$ .

5.3 Mix well and sterilize 450 mL over an 0.22  $\mu\text{M}$  filter (2.2). Use this medium during liver surgery.

5.4 Add 8 g low-endotoxin BSA to the remaining 200 mL buffer and sterilize over an 0.22  $\mu$  filter (2.2) after the BSA has been dissolved (this may take about 1 hour). Use this medium for perfusing the liver in the perfusion cabinet.

**6. DATA ANALYSIS/RECORDS: N/A**

**7. QUALITY CONTROL:**

7.1 Before using stock solutions make sure they are still clear.

**8. PROCEDURE NOTES:**

- 8.1 Since usually two IPRL experiments are performed a week, it is convenient to prepare the double amount of perfusion medium on Monday at a time. Prepare medium under sterile conditions to prevent bacteria growth.

**9. REFERENCES:**

- 9.1 Krebs Ringer bicarbonate solution for use in isolated rat liver perfusions (SOP NO. 4300-4056 [ManTech]).

**10. ADDENDA/ATTACHMENTS: N/A**

**APPENDIX C: SOP NO. PD-97-008 "Enzyme leakage from the Isolated Perfused Rat Liver"**

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## ENZYME LEAKAGE FROM THE ISOLATED PERFUSED RAT LIVER

### PURPOSE/PRINCIPLE:

Purpose: The isolated perfused rat liver (IPRL) can be used to study the role of the liver in metabolism of xenobiotica and to study their toxicity. Cytotoxicity can be evaluated using a variety of cellular function parameters. Leakage of cellular enzymes in the extracellular medium indicates that the plasma membrane has been severely damaged. Routinely measured enzymes to assess liver integrity are: lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT). LDH and ALT are localized in the cytosol whereas AST is localized in mitochondria. The percentage of enzyme-leakage is commonly used as an indicator for the extent of loss of membrane integrity. If the  $K^+$  content is also of interest, both  $K^+$  and enzyme activities can be measured in the samples prepared as described in this SOP.

Principle: Activity of the enzymes is measured as U/L. One U represents 1  $\mu$ mol of substrate converted to the product per minute at 37 °C. The activity of the enzymes is measured in perfusion medium and in liver homogenates.

**KEY WORDS:** IPRL, AST, ALT, LDH, cytotoxicity, PD, perfusion, liver, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase

### 1. SAFETY AND OPERATING PRECAUTIONS:

- 1.1 Wear protective gloves and lab coat during procedures since this work is done with biological samples.
- 1.2 Perform procedures on ice as much as possible.

### 2. EQUIPMENT/MATERIALS:

- 2.1 Microcentrifuge (Eppendorf, 5415)
- 2.2 Homogenizer (TRI-R Instruments, K43)

- 
- 2.3 Ultrasonic Homogenizer (Cole-Parmer instrument Co., 4710)
  - 2.4 Clinical analyzer (Du Pont, ACA IV discrete clinical analyzer)
  - 2.5 Clinical analyzer (Kodak, Ektachem 250)
  - 2.6 Tris-HCl
  - 2.7 Sucrose
  - 2.8 Ice bucket

**3. SPECIMEN:**

- 3.1 Liver homogenate (see Section 5.1.6)
- 3.2 Perfusion medium

**4. REAGENTS:**

- 4.1 Tris-sucrose buffer - 50 mM Tris/200 mM sucrose
  - 50 mM Tris                      3.03 g
  - 200 mM Sucrose                68.5 g
  - QS to 500 mL dH<sub>2</sub>O/pH 7.2 with 5N HCl
- 4.2 Phosphate buffered Saline (PBS)

**5. PROCEDURE:**

- 5.1 Liver:
  - 5.1.1 Chop the liver and homogenize 1 g of minced liver with 3 mL ice-cold Tris-sucrose buffer (4.1) using a Potter-Elvehjem tissue

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grinding chamber (Thomas; no. A 62247) and motor driven teflon pestle (AHT Co.; no S 756).

- 5.1.2 Transfer the homogenate in a 15-mL conical centrifuge tube and sonicate for 20 sec using the ultrasonic homogenizer (output control = 80; 20-40 = 20).
- 5.1.3 Dilute the homogenate 1:19 with Tris-sucrose buffer (4.1) in a 15-mL conical tube (e.g., 0.5 mL homogenate + 9.5 mL Tris-sucrose buffer).
- 5.1.4 Take an aliquot for protein determination (dilute this aliquot 1:4 with PBS, e.g., 25  $\mu$ L cell lysate + 75  $\mu$ L PBS). Store protein samples in freezer at  $-20^{\circ}$  C.
- 5.1.5 Take 4, 1-mL samples of the homogenate prepared under Section 5.1.3, transfer in a microcentrifuge tube and centrifuge for 10 min at 5,000 x g in the Eppendorf microcentrifuge (2.1) (speed: 14).
- 5.1.6 Transfer supernatant into an 1.5-mL microcentrifuge tube (sample A). Keep samples on ice!

**5.2 Perfusion medium from IPRL experiments**

- 5.2.1 Take 1 mL of the medium perfusing the isolated rat liver at desired time intervals (typically every 30 min) (sample B). Keep samples on ice!

NOTE: All enzyme activities in the samples are determined in the clinical pathology laboratory.

**6. DATA ANALYSIS/RECORDS:**

**6.1 Calculations:**

Enzyme leakage from the isolated perfused liver at time x:

$$E_{leak,x} = 100\% * \frac{E_{med,x}}{E_{tot}}$$

$$E_{med,x} = E_{B,x} * V_{med,x}$$

$$E_{tot} = E_{B,e} * V_{med,e} + E_A * D * W_{liv}$$

$E_{leak,x}$  = enzyme leakage at time x (%)

$E_{med,x}$  = total amount of enzyme units in medium that has leaked from liver at time x (U)

$E_{tot}$  = total amount of enzyme units in the liver at time 0 (U)

$E_{B,x}$  = enzyme activity sample B taken at time x (U/mL)

$V_{med,x}$  = medium volume at time x (mL)

$E_{B,e}$  = enzyme activity in medium at end of experiment (U/mL)

$V_{med,e}$  = medium volume at end of experiment (mL)

$E_A$  = enzyme activity of sample A (U/mL)

$D$  = dilution factor ( $4 * 20 = 80$ )

$W_{liv}$  = liver weight

6.1.1 It is assumed that 1g of liver equals 1 mL.

6.1.2 After measuring protein samples, specific enzyme activity can be calculated in U/mg protein.

$$A_{spec} = \frac{A_B}{P}$$

$A_{spec}$  = specific enzyme activity

$A_B$  = enzyme activity of sample B (U/mL)

$P$  = protein concentration (mg/mL)

## 6.2 Results:

6.2.1 Results are reported as:

AST, ALT and LDH activity in perfusion medium over time (U/mL).

Percentage LDH leakage in time as calculated in 6.a.



**7. QUALITY CONTROL:**

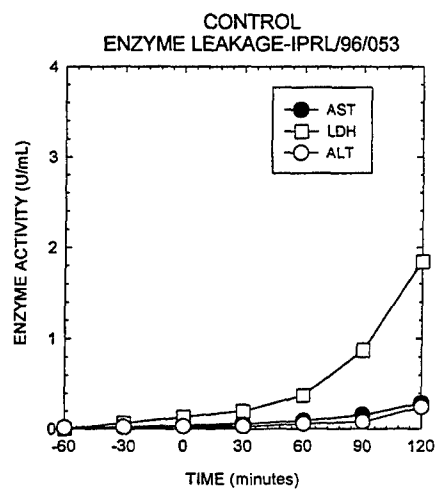
- 7.1 LDH activity in liver homogenates should be about  $4,000 \pm 500$  U/L.
- 7.2 AST activity in liver homogenates should be about  $1,500 \pm 500$  U/L.
- 7.3 ALT activity in liver homogenates should be about  $1,500 \pm 500$  U/L.
- 7.4 The percentage of LDH leakage should not exceed 20% after the liver has been three hours in the perfusion cabinet.
- 7.5 The values given here are for control livers. If enzyme activities in the perfusion medium are much higher than these values and the bile production of the liver was low, the quality of the liver was not high and the results of the experiment should not be used. If the liver was exposed to a chemical, high enzyme leakage may also indicate toxic effects of the chemical.

**8. PROCEDURE NOTES: N/A**

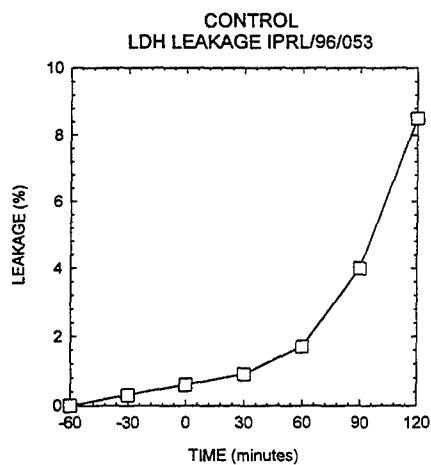
**9. REFERENCES: N/A**

**10. ADDENDA/ATTACHMENTS:**

- 10.1 Percentage LDH leakage in perfusion medium from the IPRL in time for typical control liver.
- 10.2 Changes in enzyme activity in perfusion medium over time for typical control liver.



10.1 Percentage LDH leakage in perfusion medium from the IPRL in time for typical control liver.



10.2 Changes in enzyme activity in perfusion medium over time for typical control liver.

APPENDIX D: SOP NO. PD-97-010 "Bromosulfophthalein (BSP) kinetics in the Isolated  
Perfused Rat Liver"

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**BROMOSULFOPHTALEIN (BSP) KINETICS IN THE ISOLATED PERFUSED RAT LIVER**

**PURPOSE/PRINCIPLE:**

Purpose: Bromosulfophtalein (BSP) is a well known marker for functioning of the liver. In this procedure BSP is used to study functionality of the isolated perfused rat liver (IPRL). Also, BSP kinetics are used to test and further develop a PB-PK model for the IPRL.

Principle: BSP is detected by spectrophotometric absorbance at 580 nm. It is measured in perfusion medium and bile.

**KEY WORDS:** isolated perfused rat liver, BSP, bile, liver function, PD, perfusion, liver, IPRL

**1. SAFETY AND OPERATING PRECAUTIONS:**

- 1.1 Wear a labcoat and gloves during all procedures.

**2. EQUIPMENT/MATERIALS:**

- 2.1 Bromosulfophtalein (BSP) (Sigma)  
2.2 Glass tubes, 15 mL  
2.3 1 N Sodium Hydroxide (NaOH)  
2.4 Cuvettes, 1.5 mL  
2.5 Spectrophotometer (Beckman, DU 650 spectrophotometer)  
2.6 Perfusion medium (Krebs Ringer Buffer + 4% Bovine Serum Albumin [BSA]),  
S.O.P. No. PD-97-009

**3. SPECIMEN:**

- 3.1 Rat liver
- 3.2 Perfusion medium (Krebs-Henseleit + 4% BSA)
- 3.3 Bile

**4. REAGENTS:**

- 4.1 BSP stock solution - 20 mM  
16 mg BSP per mL nanopure water
- 4.2 NaOH - 1N  
10 g NaOH per 250 mL nanopure water

**5. PROCEDURE:**

- 5.1 Liver exposure:
  - 5.1.1 Start BSP exposure 30 min after connecting the rat liver in the perfusion chamber, experimental time = -30.
  - 5.1.2 To obtain an end concentration of 20  $\mu$ M BSP, add 200  $\mu$ L stock solution (4.1) of BSP to perfusion medium (200 mL)
  - 5.1.3 Pipette perfusion medium samples of 0.5 mL in tubes at the following time points (min): -30 (blank), -28, -25, -20, -10, 0, 30, 60, 90, 120 (keep on ice)
  - 5.1.4 Collect bile at time intervals of 30 min in pre-weighed microfuge tubes and keep on ice.
- 5.2 BSP determination:

5.2.1 Medium: add 0.5 mL 1N NaOH, transfer to cuvettes and read absorbance at 580 nm.

5.2.2 Bile: mix 1 mL perfusion medium with 1 mL 1N NaOH. Add 10  $\mu$ L bile, transfer to cuvettes and read absorbance at 580 nm. Use bile collected before BSP exposure as blank.

5.2.3 Standards: prepare standards according to the following table:

End concentration ( $\mu$ M)	Perfusion medium (mL)	BSP solution
25	10	12.5 $\mu$ L stock (4.1)
20	10	10 $\mu$ L stock (4.1)
15	0.835	2.5 mL 20 $\mu$ M
10	1.25	1.25 mL 20 $\mu$ M
5	1.5	0.5 mL 20 $\mu$ M
1	1	0.25 mL 5 $\mu$ M
0 (blank)	1	---

5.2.4 Mix 0.5 mL of standard with 0.5 mL 1N NaOH and read absorbance at 580 nm. Make duplicate standards.

5.3 Clean up: since there is only a very small amount of BSP used in these experiments there is no special clean up procedure.

## 6. DATA ANALYSIS/RECORDS:

6.1 Generate a standard curve of BSP absorbance at 580 nm in SIGMA plot (Jandel Scientific) and use the slope to calculate the [BSP] in medium and bile.

## 7. QUALITY CONTROL:

7.1 To check the viability/quality of the IPRL, determine the following parameters:

7.1.1 Enzyme leakage (LDH/AST/ALT)

7.1.2 Bile production ( $\mu\text{L min}^{-1} \text{ g liver}^{-1} > 1$ )

7.1.3 Histopathology

7.2 The total amount of BSP excreted in bile should be  $> 2 \mu\text{moles}$  (desired).  
Acceptable:  $1.65 \mu\text{moles}$  (for livers weighing  $\sim 8$  grams)

**8. PROCEDURE NOTES:**

8.1 Will indicate circumstances that would vary from outlined procedure.

**9. REFERENCES: N/A**

**10. ADDENDA/ATTACHMENTS:**

10.1 BSP kinetics in the isolated perfused rat liver

- A) BSP concentration in perfusion medium. Mean  $\pm$  SD of 4 experiments
- B) BSP concentration in bile. Mean  $\pm$  SD of 4 experiments
- C) Cumulative excretion of BSP by the IPR. Mean  $\pm$  SD of 4 experiments

